pore, the fraction of the time for which the pore connects the vesicle and SBL membranes (~7-fold increase compared to ~10% for cholesterol free bilayers). Cholesterol also strongly accelerated fusion pore initiation following vesicle docking. With increasing cholesterol concentration, SNAREs open the fusion pore a greater fraction of the time than when SNAREs are absent (~2-3-fold greater with SNAREs than without). Other lipid components tested (PC, PS, PE) had a minimal effect on pore openness. Thus, we find that cholesterol promotes fusion pore opening in SNARE-mediated fusion.

171-Plat

Protein Mobility in Secretory Granules and Fusion Pore Expansion: Factors Affecting Protein Secretion

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We evaluated the importance on post-fusion discharge rates of the mobility of specific lumenal proteins within chromaffin granules in living cells. Two fluorescent proteins were investigated: tissue plasminogen activator (tPA-cerulean), which is discharged over many seconds after fusion, and NPY-cerulean, which is discharged within 200 ms. We developed a method not limited by optical resolution to measure the mobility of fluorescent proteins within individual secretory granules in living chromaffin cells. A bright flash of strongly decaying evanescent field (64 nm exponential decay constant) produced by TIRFM selectively bleached fluorophore that was proximal to the glass coverslip in individual granules (300 nm diameter). Fluorescence recovery occurred as unbleached fluorophore from distal regions of a granule diffused into the proximal regions. This experimental approach is accompanied by a new theoretical, quantitative analysis of recovery that takes into account the evanescent field depth, bleach efficiency, the limited number of total fluorophore molecules in a granule, granule diameter, and duration of bleach. The method and analysis permitted measurement of tPA mobility within chromaffin granules and a comparison of the mobilities of tPA-cerulean and NPY-cerulean. The diffusion coefficient of lumenal tPA-cerulean was 2 x 10^{-10} cm^2/s, ~1/3000 of the expected mobility in aqueous solution. The diffusion of NPY-cerulean was too fast to be resolved. A second goal bearing upon discharge rate was the measurement of fusion pore expansion using polarized TIRFM (Anantharam et al., J.Cell.Biol.2010,188:415-28.). We found that the membrane curvature change due to the fusion pore was much longer lived upon fusion of granules containing tPA-cerulean rather than NPY-cerulean. We quantitatively evaluated the relative roles of low protein mobility within a granule and a restricted fusion pore in determining the release kinetics from individual fused granules. *ANW support NIH fellowship (T32-HL-007853)*

172-Plat

Nanostructure-Induced Membrane Curvature Recruits Endocytosis Machinary in Living Cells

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Nanotechnology innovations have advanced biological science by providing new tools for probing cellular process. Whether or how cellular processes are altered upon interacting with such small scale devices are, however, not well understood. One crucial yet overlooked phenomenon is that nanostructures can induce local curvatures on plasma membrane. Modulation of local membrane curvature is known to be important in creating micro-domains for endocytosis initiation.

In the present work, we used electron-beam lithography to make patterned nanopillars arrays with controllable diameters from 60nm to 2000 nm. From both SEM and TEM studies, nanopillars were found to sufficiently induce membrane curvature in living cells. By culturing mammalian cell lines with fluorescent protein labeled clathrin and dynamin on nanopillar substrates, recruitment of these two key proteins in endocytosis machinery were found to preferentially happen on nanopillars in comparison to flat surfaces. Similar phenomenon was also found in adaptor protein AP2 and BAR domain proteins. More interestingly, when changing the nanopillar to other geometries, e.g. nanobar and nanoCUI, such recruitment was found to more correlated with positive and large curvatures. We further studied the dynamics of clathrin and dynamin on nanostructures with gradient geometry, and differential effects were observed. This work provides new insights on the curvature dependent recruitment of endocytosis machinery proteins in living cells, and demonstrates the possibility of using nanofabricated structures as a new platform for membrane curvature manipulation.

173-Plat

A Dynamin Mutant Defines a Super-Constricted Pre-Fission Step

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Dynamin is a 100 kDa GTPase that assembles around the necks of invaginated clathrin-coated pits to catalyze membrane fission during the final stages of clathrin-mediated endocytosis. Purified dynamin assembles into helical arrays on lipid templates that resemble the dense collars observed at the necks of clathrin-coated pits in vivo. Recent evidence suggests that the GTP transition state of dynamin serves as a key determinant of productive fission. The transition state stabilizes G domain dimerization, which optimally positions the catalytic machinery and thereby enhancing its intrinsic GTP hydrolysis rate. In the helical array, G domain dimerization between dynamin molecules only occurs between neighboring rungs of the helix. Thus, the architecture of the dynamin polymer ensures that assembly and stimulated turnover are tightly coupled. Here we present a three dimensional structure of a transition-state-defective mutant in the penultimate fission status at 13.5 Å resolution. This structure is tightly constricted with an inner luminal diameter of 4 nm, reaching the theoretical limit required for spontaneous fission. Computational docking of dynamin crystal structures into the 3D reconstruction suggests that a GTP ground state, and not stimulated GTP hydrolysis, drives the dynamin polymer into the super-constricted pre-fission state. Computational docking also positions the proline-rich domain (PRD) close to the G domain, which supports the notion that the PRD can modify the GTPase cycle. The surface accessibility of the PRD allows dynamin partners to bind dynamin throughout its GTPase cycle, and regulate assemble, fission and disassembly.

174-Plat

Clathrin Aggregation by Rotational Brownian Dynamics

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Endo- and exocytosis are processes associated with the transport of nutrients and proteins in to and out of living cells. Upon entering a cell these molecules are collected and encapsulated in vesicles for further transport within the cell. The central protein in the formation process of these vesicles is clathrin. Clathrins consist of three long legs that enable them self-assemble into vesicles and transport cargos within the cell.

We investigate the formation and structure of clathrin cages by means of computer simulations. To achieve this, we developed a highly coarse-grained patchy particle model by representing a clathrin protein as a rigid triskelion with interaction sites on the legs. To simulate their dynamics, we have developed a novel Brownian Dynamics algorithm[1] to describe the realistic motion of the protein. Our algorithm overcomes complications traditionally associated with rotational dynamics of anisotropic particles.

We will show results of the self-assembly of clathrin[1] into cages on a time-scale that is comparable to experimental data. In addition, the simulated cages are structurally similar to those observed by in vitro experiments and the simulations predict the clathrin interaction strength[2].

[1] I.M. Ilie, W.K. denOtter and W.J. Briels, in preparation(2013).
[2] W.K. denOtter and W.J. Briels, Traffic, 12(2011)

175-Plat

Quantifying the Dynamic Interactions between a Clathrin-Coated Pit and Cargo Molecules

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Clathrin-mediated endocytosis is a major pathway of internalization of cargo molecules in eukaryotic cells. This process involves the recruitment of cargo molecules into a growing clathrin-coated pit (CCP). However, cargo-CCP interactions are difficult to study because CCPs display a large degree of lifetime heterogeneity and the interactions with cargo molecules evolve over time. We use single-molecule total internal reflection fluorescence (TIRF) microscopy, in combination with automatic detection and tracking algorithms, to directly visualize the recruitment of individual voltage-gated potassium channels into forming CCPs in living cells. Contrary to widespread ideas, cargo often escapes